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Screening of a Large Global *Aspergillus fumigatus* Species Complex Collection by Using a Species-Specific Microsphere-Based Luminex Assay▽

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A microsphere-based Luminex assay was developed and validated for rapid identification of *Aspergillus fumigatus* from the other species within the *A. fumigatus* species complex (section *Fumigati*). This molecular tool was then employed to screen 499 clinical *A. fumigatus* species complex isolates collected from multiple medical centers throughout the world with results demonstrating the exclusive presence of *A. fumigatus*.

Recently, we designed an *Aspergillus*-specific multiplex assay using the Luminex xMAP platform (Luminex Corp., Austin, TX) targeting *Aspergillus* species above the section level (species complex) with probes directed to the internal transcribed spacer 1 region (7). Though this locus is useful for species complex-level identification, it is not helpful in differentiating individual species within the species complex such as *Aspergillus lentulus* and *Neosartorya pseudofischeri*, two clinically relevant species within the *Aspergillus fumigatus* species complex (2, 6). Identification of species within the *Aspergillus* species complex (section *Fumigati*) may be important, as previous studies have shown the existence of cryptic species whose antifungal susceptibility profile differs from that of *A. fumigatus* (1). Comparative sequence analysis of protein coding regions offers enough discrimination to differentiate individual species within the *Aspergillus* species complex (2, 8, 10). To this end, we employed the β-tubulin region to design and validate an *A. fumigatus*-specific probe for species identification using Luminex technology. Additionally, we employed this *A. fumigatus*-specific Luminex (AFSL) assay to determine the global distribution of species within the *A. fumigatus* complex by screening 499 *A. fumigatus* clinical isolates collected as part of the ARTEMIS antifungal surveillance program from 2006 to 2009 (11).

For design of the *A. fumigatus* probe, sequences of the β-tubulin region for all the *Aspergillus* species complex isolates were downloaded from GenBank (10). An *A. fumigatus*-specific probe, 5′-AG GCT ACC TCC ATG GGT TCA GCC T-3′, was designed specifically to hybridize to *A. fumigatus*; employment of this probe in the AFSL assay will yield no signal with other, non-*A. fumigatus* species (because of the lack of probe-DNA hybridization). Our rationale for designing this specific probe was based on two factors: (i) a national, multicenter study (TRANSNET [9]) demonstrated that more than 90% of the 190 *A. fumigatus* species complex isolates screened were *A. fumigatus* (5) and (ii) designing individual probes to the constantly expanding species in the *A. fumigatus* species complex whose clinical relevance is not known seemed unwarranted and would add complexity to this assay. Thus, a single *A. fumigatus*-specific probe would be cost-effective for screening a large number of isolates. The small number of isolates failing to hybridize to the probe could be directly sequenced and identified.

The *A. fumigatus*-specific probe was coupled to microspheres, and probe coupling was confirmed as previously described (7). Primers TUBF (AAT TGG TGC CGC TTT CTG G) and biotin-labeled reverse primer TUBR (AGT TGT CGG GAC GGA ATA G) were designed to amplify a 500-bp region of the β-tubulin gene (9). Forty-five *A. fumigatus* species complex isolates including 18 reference/type *A. fumigatus* species complex isolates (listed in Table 1) and 27 *A. fumigatus* clinical isolates from the culture collection of the Mycotic Diseases Branch at the Centers for Disease Control and Prevention were included as the initial test panel. The identities of all 45 isolates were confirmed by comparative sequence-based identification methods performed with a portion of the β-tubulin gene region (4). For the AFSL assay, DNA was extracted from all 45 aspergilli and subjected to PCR and Luminex probe hybridization as described previously (7). Data were acquired using the MasterPlexCT system and analyzed using the MasterPlexGT software (MiraiBio, San Francisco, CA). Median fluorescence intensity (MFI) values were generated per microsphere set, and an MFI of at least twice that of background was defined as positive for hybridization (7). Results showed that *A. fumigatus* isolates consistently generated an average MFI of 458, more than twice the background MFI (Table 1). In contrast, none of the other species tested in this study yielded detectable MFIs, thereby confirming assay specificity.

We employed the AFSL assay to screen a large clinical collection of *A. fumigatus* species complex isolates with the aim of determining the species distribution of section *Fumigati* isolates. This global culture collection consisted of a total of 499 unique *A. fumigatus* species complex isolates obtained from 62 different medical centers worldwide and represented

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TABLE 1. *A. fumigatus* species complex isolates included in the study

Isolate	Source[a]	Hybridization
Aspergillus fumigatus	CBS 542.75	Avg MFI = 458
Aspergillus lentulus[b,c]	CBS 117887	NH[d]
Neosartorya udagawae	CBS 2040	NH
Neosartorya pseudofischeri[b]	CDC IFI02-0149	NH
Aspergillus viridinutans	FRR 1266	NH
Neosartorya hiratsukae	CBS 1377	NH
Neosartorya denticulata	CBS 290.74	NH
Neosartorya multiplicata	CBS 646.95	NH
Neosartorya delicata	CBS 101754	NH
Neosartorya australiensis	CBS 117059	NH
Neosartorya neofennelliae	CBS 117186	NH
Aspergillus novofumigatus	CBS 117519	NH
Aspergillus parafumigatus	CBS 117520	NH
Aspergillus pseudofumigatus	CBS 117522	NH
Neosartorya aureola	CBS 105.55	NH
Neosartorya fischeri var. *glabra*	CBS 206.92	NH
Aspergillus unilateralis	CBS 283.66	NH
Neosartorya quadricincta	CBS 135.52	NH

[a] Reference isolates were from the CBS Fungal Biodiversity Centre, Utrecht, The Netherlands, and the CDC Mycotic Disease Branch Culture Collection.
[b] Clinical isolate.
[c] Five isolates used in the study.
[d] NH indicates "no hybridization" with an MFI value of 0.

the entire collection of *A. fumigatus* isolaes collected from 2006 to 2009. Isolates represented consecutive molds submitted as part of the ARTEMIS study that were reconfirmed at the University of Iowa as *A. fumigatus* by the use of traditional morphological methods of identification (11). The clinical isolates were obtained from a variety of sources, including sputum, bronchoscopy, and tissue biopsy specimens and were identified by phenotypic methods that included morphology and thermotolerance. This collection represented a global collection with isolates from South America (20%), North America (27%), Europe (23%), Africa (0.4%), Asia (26%), and Oceania (3%).

Results of the AFSL assay demonstrated that all of the 499 isolates screened were *A. fumigatus* (average MFI = 418). To confirm the result of the AFSL assay, 96 isolates were randomly selected and subjected to PCR/sequencing of the β-tubulin region. Comparative sequence analysis of the β-tubulin region revealed that all 96 isolates were *A. fumigatus* with 99 to 100% identity to NRRL 6113 (GenBank accession no. EF669854), substantiating the results of the AFSL assay. These results were also in keeping with the previous finding that all these isolates grew at 50°C (10), a phenotypic feature specific to *A. fumigatus* (3).

Numerous recent reports from single centers and screening studies using banked specimens and/or historical culture collections of morphologically identified *A. fumigatus* species complex isolates have detected the presence of non-*A. fumigatus* species (1, 2, 8, 12). We recently screened a large number of clinical *A. fumigatus* species complex isolates collected as a part of a nationwide fungal surveillance study (TRANSNET) and found that *A. fumigatus* was isolated from 94% of the cases studied with only 6% represented by three other species within the *A. fumigatus* species complex—*A. lentulus* (3%), *Aspergillus udagawae* (2%), and *N. pseudofischeri* (one isolate) (5). The present study is the first to determine the species distribution of a global collection of *A. fumigatus* species complex isolates. Our previous U.S.-based TRANSNET study found that non-*A. fumigatus* species were infrequently identified from clinical samples (5). Interestingly, in the present study, non-*A. fumigatus* species were not recovered from the global collection. One possible explanation for this discrepant finding may be that the non-*A. fumigatus* isolates may have geographical specificity. Although *A. lentulus* (a non-*A. fumigatus* species) appears to have a global distribution (4), the TRANSNET study found that all the *A. lentulus* isolates were recovered from only one geographical area (Seattle, WA) that was not represented in the current study (5). If true, the restriction of some aspergilli to certain locales is intriguing and warrants further investigation (5).

To summarize, we report the development of a rapid, multiplexed microsphere-based Luminex assay specific for the detection of *A. fumigatus*. Screening of a global collection of *A. fumigatus* species complex isolates demonstrated that 100% of these isolates represent one species, *A. fumigatus*.

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